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(54) Efficient Method for Regenerating Cotton from Cultured Cells

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An Efficient Method for Regenerating Cotton From Cultured Cells

Abstract

Method for producing pro-embryonic cotton cell masses in liquid suspension comprising the steps of:

(a) inducing cotton callus formation by placing cotton plant tissue on a suitable callus induction medium at about 20° to 40°C with sufficient subcultures to prevent browning;

(b) suspending up to 40 mg/ml of the callus in a suitable liquid pro-embryonic cell mass inducing medium comprising a relatively low concentration of at least one auxin until clumpy aggregates of pro-embryonic cell masses form and begin rapidly to proliferate; and

(c) transfering the rapidly proliferating clumpy aggregates of pro-embryonic cell masses to a liquid nutrient medium capable of producing smaller, more finely dispersed pro-embryonic cell masses, said nutrient medium comprising a relatively high concentration of at least one auxin.

The pro-embryonic cell masses are capable of regeneration into mature embryos, plantlets and whole plants.

5-16769/=/CGC 1314

An Efficient Method for Regenerating Cotton From Cultured Cells

Background

The present invention is directed to methods for regenerating cotton plants from cultured cells by means of somatic embryogenesis. The method is more efficient than the methods described in the prior art.

Somatic embryogenesis should be distinguished from another plant regeneration method known as organogenesis. In both techniques, explants from sources such as roots, leaves, or stems are cultured. New differentiated tissues form either from the explant directly or from undifferentiated callus tissue that develops from the explant. In organogenesis, the new differentiated tissues are roots and/or shoots. In somatic embryogenesis, on the other hand, the new differentiated tissues are bipolar structures containing connected root and shoot meristematic centers i.e. embryos. Various stages can be recognized during embryo development. These stages include those known in the art as globular, heart, torpedo and mature stages.

Under appropriate conditions, the embryos germinate and grow into plantlets, which, in turn, grow into whole plants. Somatic embryos can be produced in large quantities and are, therefore, suitable for mass propagation and cloning.

In some cases, the cells of whole plants produced by somatic embryogenesis have been found to differ genetically from the cells used to produce the embryogenic callus. A plant cell modified in this way is said to have undergone a genetic alteration known as somaclonal variation. In some cases, the new genotype produces a desirable trait. The introduction of modified genotypes through plant cell culture constitutes another use of somatic embryogenesis.

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Somatic embryogenesis may also be used to regenerate plants from cultured cells that have been selected for a particular trait. For example, cultured cells may be exposed to a phytotoxin, to which tolerance is desired. Somatic embryogenesis may then be used to regenerate plants from the cells that are best able to tolerate the phytotoxin. Such in vitro selection using cultured cells and tissues in this way has been described by Chaleff et al. in Science 223, 1148-1151 (1984).

There have been reports of regenerating cotton plants from tissue by somatic embryogenesis in the prior art. For example, Rangan et al (In Vitro, 20, 256 (1984)) reported the successful regeneration of cotton (G. hirsutum) by somatic embryogenesis at the 35th annual meeting of the Tissue Culture Association in Houston in 1984. Davidonis et al. [Plant Science Letters, 32, 89 (1983)] produced a few plants from somatic proembryoids of G. hirsutum, which were obtained from two year old callus tissue. Finer et al. [TCA Report, 17, 8 (1983)] regenerated a plant from somatic embryos of G. klotzschianum. Smith et al. [In Vitro, 13, 329 (1977)] regenerated one plantlet from the callus of a cotyledon from G. arboreum.

The methods disclosed in the prior art produce embryos on solid media, and are deficient in that they do not produce plants in the large numbers needed for commercial applications. The ability to regenerate plants from cultured cells has practical utility only if the formation of propagules and the regeneration of plants from propagules are efficient. Therefore, more efficient methods are needed for regenerating cotton plants through somatic embryogenesis.

Objects of the Invention

The objects of the present invention are methods more efficient than those of the prior art for producing somatic embryos from cultured cotton cells and regenerating plants from such embryos. Further objects of the present invention are methods for somatic embryogenesis using a cell suspension culture system.

Summary of the Invention

These and other objects of the present invention have been accomplished by providing a method for producing pro-embryonic cotton cell masses in liquid suspension comprising the steps of:

(a) inducing cotton callus formation by placing cotton plant tissue on a callus induction medium at about 20° to 40°C with sufficient subcultures to prevent browning;

(b) suspending up to 40 mg/ml of the callus in a liquid pro-embryonic cell mass inducing medium comprising a relatively low concentration of at least one auxin until clumpy aggregates of pro-embryonic cell masses form and begin rapidly to proliferate; and

(c) transferring the rapidly proliferating clumpy aggregates of pro-embryonic cell masses to a liquid nutrient medium capable of producing smaller, more finely dispersed pro-embryonic cell masses, said nutrient medium comprising at least an auxin in a concentration higher than those concentrations which are normally used in suspension culture media and in any case significantly higher than the corresponding auxin concentration in step (b).

If required, the above steps (a) to (c) can be followed by (d) developing torpedo or cotyledonous embryos from pro-embryonic cell masses of step (b) or step (c) by placing the pro-embryonic cell masses in a liquid torpedo or cotyledonous embryo inducing nutrient medium.

The pro-embryonic cell masses are capable of regenerating into mature embryos, plantlets and whole plants by germinating the embryos on an embryo germination nutrient medium

and then, if required, growing plants from the germinated embryos.

Figures

Figures 1 to 8 show the various stages of the present invention.

Fig. 1 is a photograph of callus produced by the method described in step (a) below using somatic embryos as the source. The photograph is enlarged 6.2 times. The bar of 1.2 cm represents 2 mm.

Fig. 2 is a photograph of callus produced by the method described in step (a) below using cotyledons as the source. The photograph is enlarged 22.7 times. The bar of 1.2 cm represents 0.5 mm.

Fig. 3 is a photograph of clumpy aggregates of pro-embryonic cell masses produced by the method described in step (b). The photograph is enlarged 44.4 times. The bar of 1.1 cm represents 0.25 mm.

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Fig. 4 is a photograph of finely dispersed pro-embryonic cell masses produced by the method described in step (c). The photograph is enlarged 44.4 times. The bar of 1.1 cm represents 0.25 mm.

Fig. 5 to 8 are, respectively, photographs of globular, heart, torpedo and mature embryos produced by the method of step (d) of this invention. The photographs are enlarged 2.7 times. The bar of 1.8 cm in each photograph represents 0.5 mm.

Detailed Description of the Invention

It has surprisingly been found that cotton (Gossypium spp.) embryos capable of germination and regeneration can be efficiently produced through somatic embryogenesis by developing pro-embryonic cell masses and, from them, embryos in a cell suspension culture system.

The present method permits the production, for example, in a standard 250 ml DeLong flask of about 10,000 globular embryos, from which about 1000 mature embryos and about 50 plants may be obtained. The methods of the prior art do not permit such efficiency.

The cotton plants produced in accordance with this invention may be cultivated or wild. Cultivated cotton plants are preferred.

Some examples of cultivated cotton include Gossypium hirsutum, Gossypium arboreum, and Gossypium barbadense. Gossypium hirsutum is preferred. Some varieties of G. hirsutum capable of being regenerated by the method of the present invention include Coker 310, Coker 312, Acala SJ2, Acala SJ4, Acala SJ5, Funk 519-2, and Funk 522-1. The preferred variety is Coker 310.

Step a: Embryogenic Cotton Callus

The first step in the method of this invention is to induce cotton callus formation from cotton explant tissue. Some examples of suitable cotton explant tissue include somatic embryos, mature and immature zygotic embryos, cotyledons or hypocotyls from a seedling, and young tissue from a mature plant. Somatic embryos and seedling cotyledons or hypocotyls are preferred.

Zygotic embryos, for example, may be obtained by excision from ovules. The ovules are preferably excised about 7 to 30 days after pollination, preferably about 10 to 21 days after pollination, and most preferably about 12 to 16 days after pollination.

Cotyledons and hypocotyls may be obtained from young seedlings. The seedlings are preferably between about 3 and 21 days old, more preferably between about 4 and 9 days old, and most preferably about 7 days old. Hypocotyls are sliced longitudinally and cut into convenient sections for example between 1 and 20 mm, preferably about 2 mm. Cotyledon tissue is cut into sections between 1 and 400 mm^2, preferably between 5 and 100 mm^2, and most preferably about 10 mm^2.

Somatic embryos derived from this procedure are the most preferred source for obtaining embryogenic callus according to the present method.

Somatic embryos may, for example, be obtained by using the method described above for hypocotyl and cotyledonary tissue as the explant source. Any somatic embryo taken before primary leaf expansion is suitable. The size of the somatic embryo is not critical. Preferably, the somatic embryo is less than about 5 mm in length.

Young tissue from a mature cotton plant may conveniently be obtained by excising the apical 10 cm, preferably about 5 cm, of a shoot tip. Stem and petiole tissue are sliced longitudinally and cut into the same size sections as are hypocotyls (see above). Leaf tissue is cut into the same size section as cotyledon tissue (see above).

The cotton plant tissue is placed on a suitable callus induction medium at about 20° to 40°C, preferably 23° to 35°C, more preferably about 31°C. Any medium capable of inducing callus from the tissue may be used in the method of the invention. The medium may be liquid or solid, although a solid medium is preferred since it is more convenient.

One medium capable of inducing callus under the conditions of the invention contains inorganic salts, vitamins, a carbon source, an auxin, and a cytokinin. The medium is adjusted to a pH between 3.5 and 7.5, preferably between 4.5 and 6.5, and most preferably about 5.7.

Any inorganic salts and vitamins capable of contributing to callus induction are suitable. Some examples of suitable inorganic salts and vitamins include those described by Murashige and Skoog in Physiol. Plant, 15, 473-497 (1962) (MS) and Gamborg et al. in Exp. Cell Res., 50, 151-158 (1968) (B-5). Another example is a modification of MS or Gamborg's B-5 media described by Cheng et al., Plant Sci Lett., 19, 91-99 (1980). The preferred inorganic salts are MS inorganic salts. The preferred vitamins are Gamborg's B-5 vitamins.

The carbon source may be any carbon source on which callus can be grown. The preferred carbon sources include sugars and derivatives of sugar. The preferred sugars are glucose and sucrose. It is especially desirable to initiate callus in a callus induction medium containing glucose in order to reduce browning of the tissue, and then to transfer the callus to a callus induction medium containing sucrose.

The concentration of the carbon source is 5 to 60 g/liter, preferably about 30 g/liter.

The auxin present in the callus induction medium may be any auxin capable of inducing callus. Some suitable auxins include α-naphthaleneacetic acid, picloram, 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid, indole-3-butyric acid, indole-3-lactic acid, indole-3-pyruvic acid, indole-3-acetic acid, and p-chlorophenoxyacetic acid. A preferred auxin is α-naphthaleneacetic acid.

Any concentration of auxins capable of inducing callus formation may be used in the method of the invention. A suitable concentration is 0.1 to 10 mg/liter. A preferred concentration is about 2 mg/liter, especially when the auxin is α-naphthaleneacetic acid.

The cytokinin present in the callus induction medium may be any cytokinin capable of inducing callus. Some suitable cytokinins include kinetin, 6-benzyladenine, 2-isopentenyladenine, and zeatin. A preferred cytokinin is kinetin.

Any concentration of cytokinin capable of inducing callus formation may be used in the method of the invention. Suitable concentrations are 0.1 to 10 mg/liter. A preferred concentration is 1 mg/liter, especially when the cytokinin is kinetin.

If the medium is solid, it contains a component that causes solidification, for example about 0.8 % agar such as Agar Noble (Difco) or about 0.8 % agarose. (All percents in this specification are by weight).

The tissue is cultured on the callus induction medium for a period of time sufficient for the callus to form. For example, tissue may be cultured on a callus induction medium containing glucose as the carbon source. A five week induction period is typical. Subcultures are performed as necessary to prevent browning. Weekly subcultures are preferred.

The callus that forms may be unorganized, or may contain pro-embryonic cell masses, embryogenic callus and/or embryos. Normally, when hypocotyls or cotyledons are used as the explant source, the callus appears to be unorganized. When somatic embryos are used as the explant source, at least part of the callus appears to comprise embryogenic callus, which is characterized by a light yellow color and nodulation. Photographs of calli produced by step (a) from somatic embryos and from cotyledons are reproduced in fig. 1 and fig. 2, respectively.

The resulting callus may then advantageously be transferred to a callus subculture medium, which is similar to a callus induction medium except that it contains sucrose as the carbon source, for a period of time up to 5 months. One month, or two months with a subculture into fresh medium after one month, on a sucrose-containing callus induction medium is preferred.

The callus may be induced in the dark, but is preferably induced in the light. The light may have an intensity of, for example, 0.5 to 150 $\mu Em^{-2}s^{-1}$ (= 41.75 to 12525 lx).

Step b: Clumpy Aggregates of Pro-embryonic Cell Masses

The callus from step (a) is suspended in a liquid medium promoting the development of pro-embryonic or proliferating embryonic cell masses. It is important for the cell density to be low. Therefore, not more than 40 mg of callus/ml of culture medium, preferably not more than 15 mg of callus/ml of culture medium and more preferably not more than 5 mg of callus/ml of culture medium is suspended.

The medium useful in step (b) may be any medium capable of inducing pro-embryonic cell masses. The medium comprises inorganic salts, vitamins, a carbon source, and an auxin. The medium may also include organic nitrogen sources, cytokinins, amino acids and other addenda such as casein hydrolysate or coconut water.

The inorganic salts and vitamins may be the same as in step (a) (supra). MS inorganic salts and B-5 vitamins are preferred.

The carbon source may be the same as in step (a) (supra). Sucrose is preferred. The concentration of the carbon source is 0.1 to 100 g/liter. About 20 g/liter is preferred, especially when the carbon source is sucrose.

The auxin may be selected from the auxins used in step (a). The preferred auxins are 2,4-dichlorophenoxyacetic acid and picloram. Picloram is most preferred.

The concentration of the auxin in step (b) is relatively low. The exact concentration depends on the specific auxin used. The relatively low auxin concentration is generally similar to that usually used in suspension culture media and is significantly lower than the corresponding auxin concentration used in step (c). When picloram is the auxin used in step (b), the concentration is 0.01 to 5 mg/liter, preferably 0.1 to 1 mg/liter, and most preferably about 0.5 mg/liter. When 2,4-dichloro-

phenoxyacetic acid is the auxin used in step (b), the concentration is 0.01 to 0.5 mg/liter, preferably 0.05 to 0.25 mg/liter, and most preferably about 0.1 mg/liter.

The induction of pro-embryonic cell masses is preferably carried out in an aerated medium at a temperature between 20° and 35°C, preferably between 22° and 33°C and most preferably between 25° and 31°C. One may aerate the medium by any method known in the art, for example by shaking. Step (b) may be carried out in the dark or in light up to about 75 $\mu Em^{-2}s^{-1}$ (= 6262,5 lx), preferably between 5 and 10 $\mu Em^{-2}s^{-1}$ (= 417,5 and 835 lx).

The callus is maintained in the medium preferably without subculture until clumpy aggregates of pro-embryonic cell masses form and begin to proliferate rapidly. The onset of rapid proliferation usually takes between 3 and 8 weeks, more typically between 5 and 7 weeks. During the induction period, the medium may be replaced by fresh medium, although it is preferable not to disturb the medium during this period.

The change from callus to clumpy aggregates of pro-embryonic cell masses will be readily apparent to those of ordinary skill in the art of plant tissue culture. It is distinguishable by the light yellow color and clumpy nature of the pro-embryonic cell masses. A photograph of such cell masses is reproduced in fig. 3.

Once the clumpy aggregates of pro-embryogenic cell masses begin to proliferate rapidly, they may be introduced directly into the medium described in step (c), or they may be subcultured in order to prevent browning. Subculturing every 3 to 7 days, preferably every 5 to 7 days, is convenient. The cell masses survive without subculture for about fourteen days.

Step c: Finely Dispersed Pro-embryonic Cell Masses

The clumpy aggregates of pro-embryonic cell masses from step (b) are transferred to a liquid medium that is capable of causing the clmpy aggregates of pro-embryonic cell masses to become finely dispersed. The medium may be similar to that described in step (b), except that the

medium of step (c) comprises a relatively high concentration of an auxin. The auxin may be any auxin used in step (a). The preferred auxins are 2,4,5-trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid. The most preferred auxin is 2,4-dichlorophenoxyacetic acid.

The concentration of auxin depends on the particular auxin used. The auxin concentration in the medium of step (c) is generally higher, or at least at the high end of the range of, than concentrations that are usually used in suspension culture media, and in any event is significantly higher than the corresponding auxin concentration used in step (b).

For example, when 2,4-dichlorophenoxyacetic acid is the auxin in the medium of step (c), the concentration may be about 0.5 to 100 mg/liter, preferably 1 to 10 mg/liter, and most preferably about 2.5 to 7.5 mg/liter.

Except for the concentration and possibly the identity of the auxin, the medium, temperature, and amount of light in step (c) may be the same as that described in step (b).

The conditions of step (c) are maintained until the clumpy aggregates of pro-embryonic cell masses become smaller, more finely dispersed pro-embryonic cell masses. The appearance of the smaller, more finely dispersed pro-embryonic cell masses will be readily apparent to those skilled in the art. These cell masses are characterized by their yellow color, smooth surface, intermediate density and small size. A photograph of the cell masses is reproduced in fig. 4. The change to the smaller, more finely dispersed cell masses usually occurs within 6 weeks, more typically 2 weeks.

The culture of the small finely dispersed pro-embryonic cell masses may be maintained indefinitely, and may be subcultured so as to maintain active growth. It is convenient to subculture, for example, every 3 to 28 days, preferably every 5 to 10 days.

Step d: Mature Embryos

The smaller, more finely dispersed pro-embryonic cell masses are added to a medium that induces the development of mature embryos. The medium is preferably a liquid.

Embryos pass through a number of developmental stages before they mature and are able to germinate. These stages include globular, heart, torpedo and mature stages. The names of the stages are based on the approximate shapes of the embryos.

The medium useful in step (d) may be any medium that induces the development of mature embryos. One useful medium comprises inorganic salts, vitamins, a carbon source and an organic compound containing reduced nitrogen.

The salts and vitamins and concentrations thereof may be the same as those described in step (a). The carbon source may also be one of the carbon sources described in step (a). The concentration of the carbon source is about 1 to 10 g/liter preferably about 2 to 6 g/liter. A preferred carbon source is sucrose.

The organic compound containing reduced nitrogen may be any such compound which, when added to the medium of step (d), induces the development of mature embryos. The preferred compounds are amino acids. A preferred amino acid is glutamine.

The concentration of the organic source of free nitrogen depends on the particular compound used. An effective concentration of glutamine as the organic source of reduced nitrogen is 2 to 260 mM, preferably 5 to 100 mM, and most preferably 10 to 50 mM.

The medium of step (d) may contain an auxin. Auxins are desirable during the early stages of embryo development, but not during the later stages. Therefore, if auxins are present at all, they are preferably present only until the heart stage of development. Then, the embryos are transferred to a medium that contains no auxin.

If present, the auxin concentration may be 0.01 to 0.1 mg/liter.

The auxin may be one of the auxins useful in step (a). The preferred auxins are picloram and 2,4-dichlorophenoxyacetic acid.

The embryos may be cultured in the medium of step (d) at temperatures of 20° to 35°C in the dark or in light. The intensity of the light may be, for example, 5 to 75 $\mu Em^{-2}s^{-1}$ (= 6262.5 lx).

The embryos are maintained in the medium of step (d) until the embryos have matured into torpedo or mature states. Those skilled in the art of plant tissue culture will be able to recognize the globular, heart, torpedo and mature embryos as they form. Photographs of these embryos are reproduced in fig. 5 to 8. The embryos mature, typically, in 2 to 5 weeks, usually in 3 to 4 weeks. It is usually unnecessary to subculture the embryos or to transfer the embryos to fresh medium, except possibly to change from an auxin-containing medium to a medium not containing an auxin at the heart stage.

Step e: Germination

The mature embryos are placed on a solid medium capable of inducing germination. The medium comprises inorganic salts, vitamins, and a carbon source. The medium is solidified with a suitable solidifying agent such as Gelrite* (Kelko, San Diego, California), agarose or agar.

The inorganic salts may be those described in step (a), modified so that nitrate is present at high concentration while ammonium is either absent or is present at very low concentration. The concentration of nitrate may be 20 to 60 mM, preferably 30 to 60 mM, more preferably 35 to 45 mM. The concentration of ammonium ion should be no greater than 5 mM.

The source of carbon is preferably a sugar. The preferred sugar is sucrose. The concentration of the carbon source depends on the particular carbon source used. For example, when sucrose is the carbon source, the concentration is 0.1 to 6 % by weight, preferably 0.5 to 4 %, more preferably 1 to 3 %.

*Trade-Mark

An organic compound containing reduced nitrogen is optionally present in the medium of step (e). The organic compound is preferably an amino acid or a mixture of amino acids capable of supporting germination. Preferred amino acids or mixtures thereof are glutamine or casein hydrolysate.

The concentration of the organic source of reduced nitrogen depends on the specific compound used. For example, when the compound is glutamine, the concentration may be 2 to 50 mM, preferably 5 to 30 mM, more preferably 10 to 20 mM. When the compound is casein hydrolysate or modified casein hydrolysate, the concentration is 100 to 3000 mg/liter, preferably 1000 to 2800 mg/liter, more preferably 1500 to 2500 mg/liter.

Preferably, germination occurs on a medium containing an organic nitrogen source until shoots develop. The embryos are then transferred to a medium containing no organic nitrogen source for elongation of roots.

The density of embryos in the medium is limited to a density less than that which causes development to be self-inhibitory. Suitable densities include 1 to 100 embryos in a 9 cm petri disk containing about 10 to 75 ml of medium, preferably 25 to 50 ml of medium, and most preferably about 35 ml of medium.

The medium or media of step (e) are maintained at 20° to 30°C. Preferably, the temperature is about 25°C.

Some light is necessary in step (e). An intensity of light between 5 and 150 $\mu Em^{-2}s^{-1}$ (= 417.5 to 12525 lx), preferably between 10 and 75 $\mu Em^{-2}s^{-1}$ (= 835 to 6262.5 lx), is suitable.

The embryos are maintained on the medium or media of step (e) until the embryos have germinated, typically 1 to 20 days, usually 2 to 4 days. Those skilled in the art will know when the embryos have germinated.

Step f: Plants

Following germination, the plantlets are transferred to soil for growth into plants. The transferred plants are initially covered with a glass to maintain high humidity. After one week under glass, no special treatment of the plantlets or of the plants is necessary.

Utility - Propagation

Mature embryos may be used for mass propagation and cloning. This entails either germinating the embryos and transplanting the plantlets to soil, to other growth substrates, or other plant growth environments. Mature embryos may also be enclosed in an artificial seed coat and planted as "somatic seeds". Mass propagation and cloning is beneficial if hybrid parents or a hybrid itself needs to be mass produced.

Cells, pro-embryos, embryos, plantlets and plants may be analyzed at any time during the stages described above in order to determine whether any new trait is present as a result of genetic alteration. The trait may be a useful in vitro or in planta trait. Some examples of useful traits include phytotoxin tolerance, drought tolerance, cold tolerance, disease tolerance, etc.

The cells of steps (a), (b) and (c) may also be subjected to tissue culture methods capable of producing cells or plants having desirable properties, such as herbicide tolerance. Some examples of such methods include, for example, Chaleff and Ray in Science, 223, 1148-1151 (1984).

Examples

Table I - Media

All media contain Murashige and Skoog inorganic salts and Gamborg's B-5 vitamins, are adjusted to pH 5.7, and have the following composition (mg/liter):

Macronutrients	
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
KNO_3	1900
NH_4NO_3	1650
$CaCl_2 \cdot 2H_2O$	440

Micronutrients	
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	15.6
$ZnSO_4 \cdot 7H_2O$	8.6
$NaMoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CaCl_2 \cdot 6H_2O$	0.025
KI	0.83
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3

Vitamins	
Thiamine•HCl	10
Pyridoxine•HCl	1
Nicotinic acid	1
Myo-Inositol	100

In addition, the various media have the following components:

Medium #	Additional Components
1	20 g/liter sucrose, 0,6 % agar Noble (Difco)
2	30 g/liter glucose, 2 mg/l α-naphthaleneacetic acid 1 mg/liter kinetin, 0,8 % agar Noble
3	30 g/liter sucrose, 2 mg/l α-naphthaleneacetic acid 1 mg/liter kinetin, 0,8 % agar Noble
4	20 g/liter sucrose, 0,5 mg/l picloram
5	20 g/liter sucrose, 5 mg/l 2,4-dichlorophenoxyacetic acid
6	20 g/liter sucrose, 15 mM glutamine

Media at 25°, 28° and 31°C refer, in addition to the temperature, to a photoperiod of 16 hours light/8 hours dark at a light intensity of 20 $\mu Em^{-2}s^{-1}$ (= 1670 lx).

Example 1: Seed Sterilization and Planting

Seeds of cotton (Gossypium hirsutum var. Coker 310) are delinted by placing seed in concentrated H_2SO_4 for 2 min. Seeds are then washed 4 times with sterile, distilled water, dipped in 95 % ethanol, flamed and planted on Medium #1 at 31°C.

Example 2: Callus induction

Seven days following planting, seedling hypocotyls are excised, sliced longitudinally, cut into 2 mm sections and placed on Medium #2 at 31°C. Hypocotyl sections (2 mm) are transferred weekly to fresh Medium #2 and these cultures are also maintained at 31°C. Following 4 weekly transfers to Medium #2, callus tissue proliferating on the hypocotyl sections is removed from the original explant and placed on Medium #3 at 31°C. The callus is transferred to fresh Medium #3 after one month and maintained for an additional 1 to 2 months.

Example 3: Suspension Culture Initiation

For initiation of suspension cultures, 100 g of callus tissue is placed into 35 ml of Medium #4 in a 125 ml DeLong flask. Suspensions are rotated for 6 weeks at 140 rpm (rounds per minute), and 28°C, at which time they begin rapidly to proliferate.

Example 4: Embryo Development and Plant Regeneration

The embryos that form in Medium #4 proliferate even faster following replacement of Medium #4 by Medium #5. This embryogenic suspension is divided and subcultured every 3 to 7 days into fresh Medium #5. For development of embryos proliferating in Medium #5, the embryos are washed with, and then placed into, Medium #6. Three to four weeks following transfer to Medium #6, the mature embryos are placed on a solid medium at 25°C. The solid medium consists of a modified MS medium containing MS salts with 40mM KNO_3 in place of KNO_3 and NH_4NO_3, B-5 vitamins, 2 % sucrose, 15mM glutamine, and solidified with 0.2 % Geltrite (pH 5.7). Embryos are placed in petri dishes at 25°C. Shoot development is sporadic on this medium and root elongation is enhanced with the transfer of the embryos to the above modified MS medium without glutamine. Germinating embryos are then planted in vermiculite in pots and covered with a beaker (25°C). After plantlets are established in

vermiculite, the beaker is removed. Following one week at 28°C, the plantlets are placed in the greenhouse for further development into plants.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for producing pro-embryonic cotton cell masses in liquid suspension comprising the steps of:

(a) inducing cotton callus formation by placing cotton plant tissue on a callus induction medium at about 20° to 40°C with sufficient subcultures to prevent browning;

(b) suspending up to 40 mg/ml of the callus in a liquid pro-embryonic cell mass inducing medium comprising a relatively low concentration of at least one auxin until clumpy aggregates of pro-embryonic cell masses form and begin rapidly to proliferate; and

(c) transfering the rapidly proliferating clumpy aggregates of pro-embryonic cell masses to a liquid nutrient medium capable of producing smaller, more finely dispersed pro-embryonic cell masses, said nutrient medium comprising at least an auxin in a concentration higher than those concentrations which are normally used in suspension culture media and in any case significantly higher than the corresponding auxin concentration in step (b).

2. A method for producing mature cotton embryos in liquid suspension wherein said method comprises in addition to the method according to claim 1 the step of

(d) developing torpedo or cotyledonous embryos from the pro-embryonic cell masses of step (b) or step (c) by placing the pro-embryonic cell masses in a liquid torpedo or cotyledonous embryo inducing nutrient medium.

3. A method for producing cotton plantlets wherein said method comprises in addition to the method according to claim 2 the step of

(e) germinating the embryos on a embryo germination nutrient medium.

4. A method for producing cotton plants wherein said method comprises in addition to the method according to claim 3 the step of

(f) growing plants from the germinated embryos.

5. The method of claim 1, 2, 3 or 4 wherein the cotton is Gossypium hirsutum.

6. The method of claim 1, 2, 3 or 4 wherein the cotton plant tissue in step (a) is cotyledon or hypocotyl tissue.

7. The method of claim 1, 2, 3 or 4 wherein the auxin in step (b) is picloram present in a concentration of 0.1 to 5 mg/liter.

8. The method of claim 1, 2, 3 or 4 wherein the auxin in step (b) is 2,4-dichlorophenoxy-acetic acid at a concentration of 0.01 to 0.5 mg/liter.

9. The method of claim 1, 2, 3 or 4 wherein step b is conducted for 3 to 8 weeks.

10. The method of claim 1, 2, 3 or 4 wherein up to 15 mg callus/ml is suspended in the medium of step (b).

11. The method of claim 1, 2, 3 or 4 wherein the auxin of step (c) is 2,4-dichlorophenoxy-acetic acid at a concentration of 0.5 to 100 mg/liter.

12. The method of claim 2, 3 or 4 wherein the medium of step (d) comprises inorganic salts, vitamins, a carbon source and an organic compound containing reduced nitrogen.

13. The method of claim 12 wherein the compound containing reduced nitrogen is glutamine at a concentration of 2 to 250 mM.

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FIG. 1

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FIG. 2

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FIG. 3

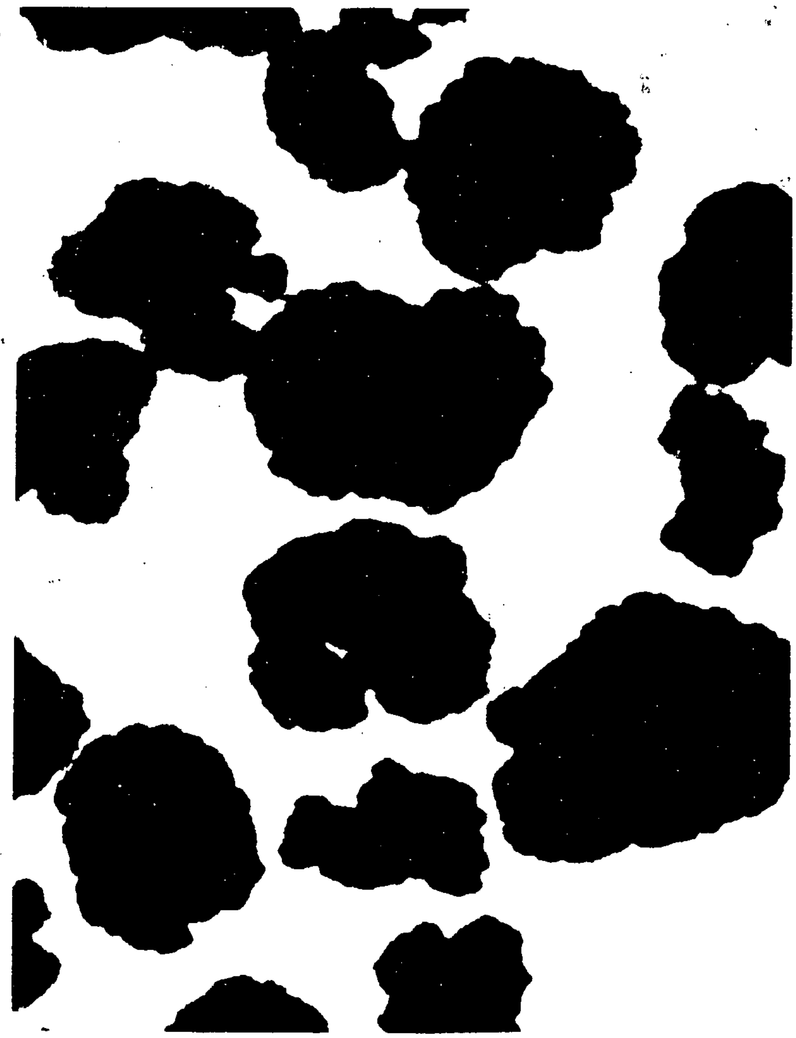

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FIG. 4

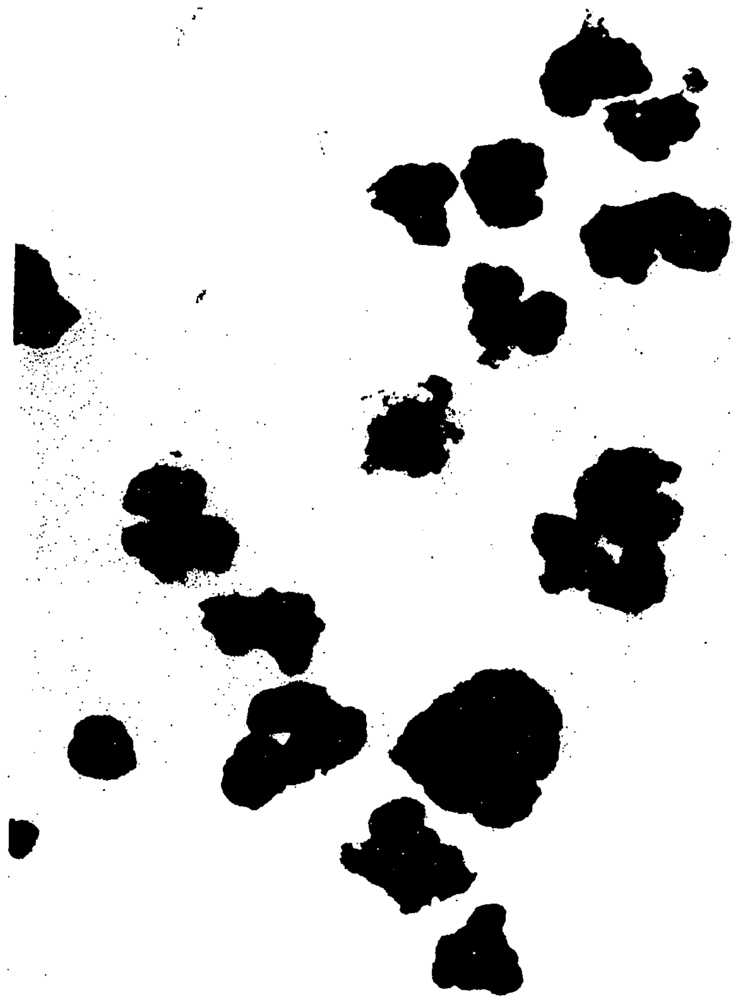

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FIG.5

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FIG. 6

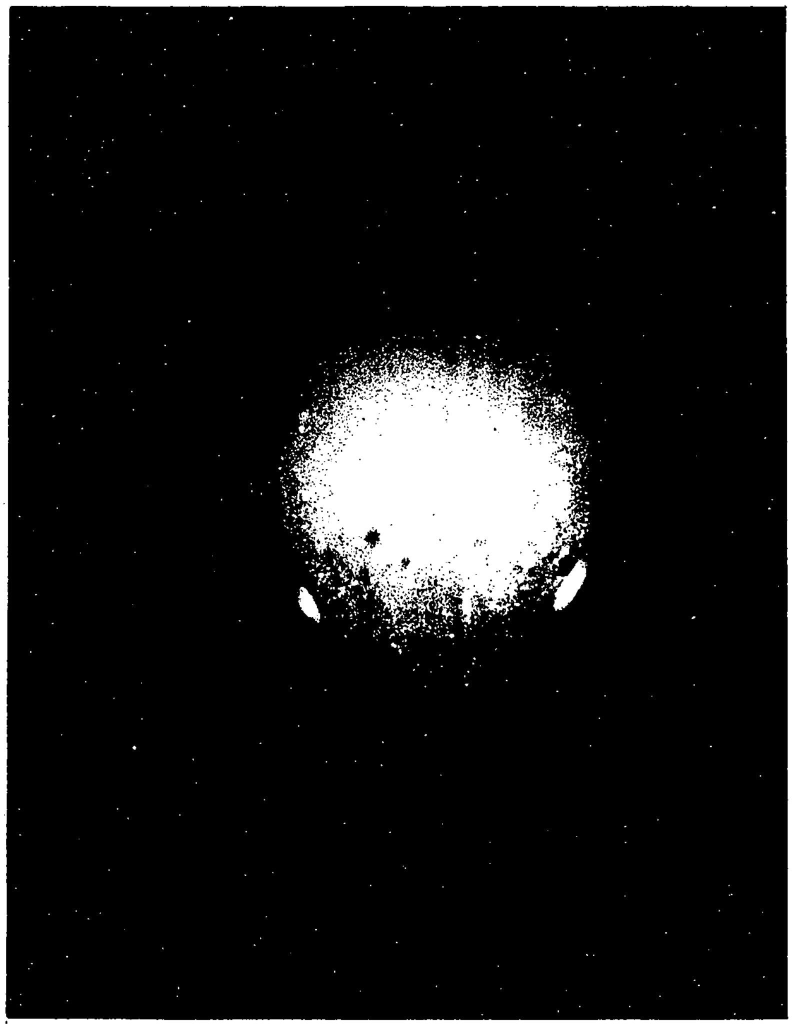

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FIG. 7

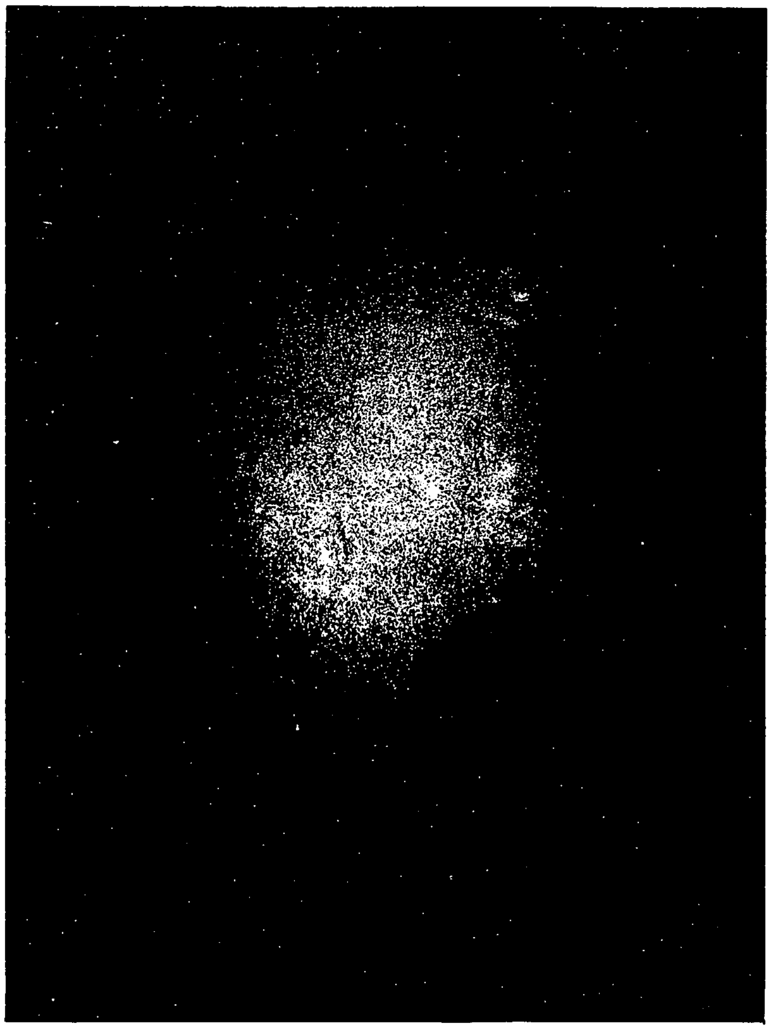

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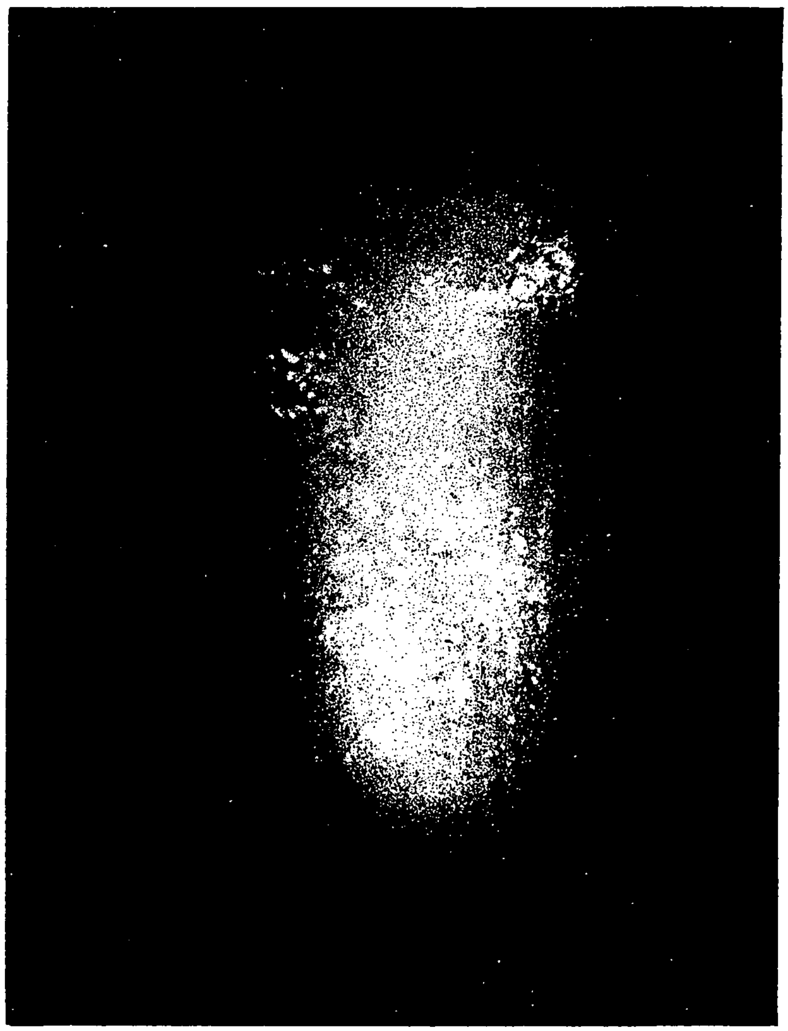
FIG. 8



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